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Immunopathology and Infectious Diseases

Rapid Myeloid Cell Transcriptional and Proteomic Responses to Periodontopathogenic *Porphyromonas gingivalis*

Salvador Nares,*† Niki M. Moutsopoulos,* Nikola Angelov,*‡ Zoila G. Rangel,§ Peter J. Munson,§ Neha Sinha,* and Sharon M. Wahl*

From the Oral Infection and Immunity Branch, National Institute of Dental and Craniofacial Research, and the Division of Computational Bioscience,§ Center for Information Technology, National Institutes of Health, Bethesda, Maryland; the Department of Periodontology,† University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; and the Department of Periodontics,‡ Loma Linda University, Loma Linda, California*

Long-lived monocytes, macrophages, and dendritic cells (DCs) are Toll-like receptor-expressing, antigen-presenting cells derived from a common myeloid lineage that play key roles in innate and adaptive immune responses. Based on immunohistochemical and molecular analyses of inflamed tissues from patients with chronic destructive periodontal disease, these cells, found in the inflammatory infiltrate, may drive the progressive periodontal pathogenesis. To investigate early transcriptional signatures and subsequent proteomic responses to the periodontal pathogen, *Porphyromonas gingivalis*, donor-matched human blood monocytes, differentiated DCs, and macrophages were exposed to *P. gingivalis* lipopolysaccharide (LPS) and gene expression levels were measured by oligonucleotide microarrays. In addition to striking differences in constitutive transcriptional profiles between these myeloid populations, we identify a *P. gingivalis* LPS-inducible convergent, transcriptional core response of more than 400 annotated genes/ESTs among these populations, reflected by a shared, but quantitatively distinct, proteomic response. Nonetheless, clear differences emerged between the monocytes, DCs, and macrophages. The finding that long-lived myeloid inflammatory cells, particularly DCs, rapidly and aggressively respond to *P. gingivalis* LPS by generating chemokines, proteases, and cytokines capable of driving T-helper cell lineage polarization without evidence of corresponding immunosuppressive pathways highlights their prominent role in host defense and progressive tissue pathogenesis. The shared, unique, and/or complementary transcriptional and proteomic profiles may frame the context of the host response to *P. gingivalis*, contributing to the destructive nature of periodontal inflammation. ***(Am J Pathol 2009, 174:1400–1414; DOI: 10.2353/ajpath.2009.080677)***

Innate and adaptive host defense mechanisms develop to counter infection and spread of bacterial, viral, and fungal organisms. In innate immunity, recognition of highly conserved pathogen-associated molecular patterns (PAMPs) by transmembrane host pattern-recognition receptors such as Toll-like receptors (TLRs) and cytoplasmic sensors including nucleotide-oligomerization domain (Nod)-like receptors initiate signaling cascades driving antimicrobial host defense.[1,2] PAMPs like lipopolysaccharide (LPS), a structural component of Gram-negative bacterial cell walls, form a LPS-TLR complex leading to activation of transcriptional programs including nuclear factor (NF)-κB- and Jun/Fos-targeted expression of inflammatory mediators critical in host defense and stimulation of adaptive immunity, but in excess, detrimental to the host.

In the oral cavity, immune-mediated destruction of the supporting tissues surrounding the dentition is a characteristic feature of chronic inflammatory periodontal disease,[3,4]

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Address reprint requests to Dr. S.M. Wahl, Building 30, Rm 320, 30 Convent Dr. MSC 4352, NIDCR, NIH, Bethesda, MD 20892-4352. E-mail: smwahl@dir.nidcr.nih.gov.

one of the most prevalent of infectious diseases. The infectious component central to the etiology of periodontal disease entails colonization of the oral niche with Gram-negative bacteria, including *Porphyromonas gingivalis*, a nonspore forming, nonmotile, obligate anaerobe strongly implicated in the pathogenic sequelae.[5] Elevated levels of *P. gingivalis* are present in periodontal lesions and are significantly reduced by successful therapy.[6] Primarily considered an oral pathogen, a significant body of emerging evidence now supports an association with systemic conditions such as coronary artery disease, diabetes, stroke, and preterm birth.[7]

P. gingivalis features numerous intensely studied structural and secreted components capable of interacting with the host immune system. Among its virulence factors, fimbrial proteins bind to and trigger epithelial TLR2,[8] whereas *P. gingivalis* LPS activates TLR2- and/or TLR4-expressing cells.[9–13] As a result of its unique structural and chemical properties, *P. gingivalis* LPS exhibits lower levels of endotoxic potency compared with classic enterobacterial preparations such as *Escherichia coli* LPS, perhaps because of lower binding affinity to host LPS-binding protein as well as its dual recognition by TLR2 and TLR4.[14]

An additional possibility is that *P. gingivalis* LPS may interact with different cell populations to orchestrate a smoldering inflammatory response. Beyond the initial encounter with $TLR2^+$ epithelial cells, *P. gingivalis* and its LPS may differentially trigger $TLR2^+$ and $TLR4^+$ cells within the context of inflamed oral mucosa to secrete inflammatory molecules, which underlie ensuing *P. gingivalis*-driven pathogenesis. Among these targeted populations are innate immune cells of myeloid origin, including recruited peripheral blood monocytes, immature and mature DCs, and differentiated macrophages, yet little is known of their differential responses to inflammatory stimuli, such as *P. gingivalis*. Prior studies have examined *P. gingivalis* stimulation of primary human monocyte or macrophage populations,[15] but this has not been examined in these populations relative to one another, nor linked with the central innate immune population of myeloid DCs. Given the common lineage of these myeloid populations and their seminal, but discrete roles in innate and adaptive immunity, we explored their shared and/or differential responses to *P. gingivalis* LPS as a means to understand their respective contributions in systemic and locally mediated diseases and in guiding T-helper cell recruitment and lineage bias. Importantly, both convergent and divergent transcriptional and proteomic profiles to *P. gingivalis* LPS were identified as a basis to begin unraveling the complex inflammatory response to microbial challenge and to identify potential targets in interrupting the chronic destructive nature of periodontal disease and/or its systemic manifestations.

Materials and Methods

Patient Selection, Tissue Collection, and Transcriptional Profile Analysis

Periodontal tissues were harvested from systemically healthy adult patients undergoing routine oral surgery procedures, after informed consent (institutional review board, OSR58034, Loma Linda University School of Dentistry). Diseased tissue samples were harvested from chronic periodontitis patients, diagnosed according to the latest American Academy of Periodontology criteria.[16] Inclusion criteria for diseased tissues were evidence of inflammation (bleeding on probing) and loss of tooth-supporting structures (clinical attachment loss >4 mm and radiographical bone loss).[16] Healthy tissue samples displayed no visible inflammation or loss of attachment and were collected during cosmetic oral surgery procedures. All tissues were bisected and processed for histology or isolation of RNA using TRIzol reagent (Invitrogen, Carlsbad, CA).

Immunohistochemistry

Gingival samples were fixed in 10% neutral-buffered formalin and embedded in paraffin. Serial 5- to 7-μm sections were stained with hematoxylin and eosin (H&E) for histopathology or incubated with monoclonal antibodies to CD68 (monocyte/macrophage; DAKO, Carpinteria, CA), CD1a (DCs) and CD3 (T cells) (Abcam, Cambridge, MA), interleukin (IL)-17 (Santa Cruz Biotechnology, Santa Cruz, CA), interferon (IFN)-γ, IL-4 (Abcam, Cambridge, MA) or an irrelevant isotype as described.[17]

Myeloid Cell Preparation and Stimulation

Human peripheral blood mononuclear cells obtained from healthy volunteers at the Department of Transfusion Medicine (National Institutes of Health, Bethesda, MD) were diluted in endotoxin-free phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (BioWhittaker, Walkersville, MD) and density-sedimented on lymphocyte separation medium (ICN Pharmaceuticals, Aurora, OH). Monocytes were purified from the mononuclear cell layer using centrifugal elutriation[18] and divided into three groups for immature monocytes and generation of macrophages and DCs as described[19,20] in three independent experiments with cells from three donors. Freshly isolated monocytes were used immediately after elutriation, whereas donor-matched macrophage and DC studies were initiated after confirmation of differentiation on day 7. The viability of each cell type was >95% as measured by trypan blue exclusion and phenotype was confirmed with the following fluorochrome-conjugated antibodies: CD14, HLA-DR, DC-SIGN, CD3, CD19, CD1a, CD68, TLR2, and TLR4 (Becton Dickinson, Sunnyvale, CA), before stimulation as measured by flow cytometry (FACSCalibur, Becton Dickinson). Cell aliquots were left untreated (medium control) or stimulated with 100 ng/ml of *P. gingivalis* LPS (American Type Culture Collection no. 33277, generous gift from Richard Darveau, University of Washington, Seattle, WA)[9] which contained <0.1% protein contamination[9] for 1 hour or stimulated in parallel with 100 ng/ml of *E. coli* LPS (055:B5; Sigma, St. Louis, MO). Additional cultures were exposed to intact *P. gingivalis* at cell to bacteria ratios of 1:10 and 1:5 for 30 minutes and processed for total RNA or protein. In separate studies, *P. gingivalis* bacteria were washed in PBS, incubated in fluorescein isothiocyanate (100 μg/ml for 30 minutes),[21] and

washed in cell culture media before being added to macrophage monolayers for indicated times at room temperature. The cultures were examined for bacterial binding using a Zeiss fluorescence microscope (Carl Zeiss, Thornwood, NY).

Myeloid Cell Transcriptional Profile Analysis

The cells were lysed and total RNA isolated using TRIzol reagent and further purification was performed using RNeasy mini columns (Qiagen, Valencia, CA) followed by assessment of RNA integrity using the 2100 Bioanalyzer (Agilent, Foster City, CA). Preparation of biotin-labeled cRNA, hybridization, and scanning were performed according to protocol (Affymetrix, Santa Clara, CA) as described.[19] Affymetrix GCOS version 1.2 software was used to calculate signal and present call values that were stored in the NIHLIMS, a database for storage and retrieval of chip data maintained at the National Institutes of Health. The signal values for the 18 chips were subjected to S10 quantile-normalizing transformation.[19] Principal components analysis on the S10-transformed data were also performed to permit the visualization of the samples in bivariate plots of the low-order principal components, to detect possible outliers and to provide a global view of the study results. Statistical tests and data calculations were with MSCL Analyst's Toolbox (*http://abs.cit.nih.gov/main/geneexpression.html*).

A three-level, one-way blocked analysis of variance compared the baseline expression levels of monocytes (MON control), dendritic cells (DC control), and macrophages (MAC control). Probesets, which manifest significant (FDR, <10%), very substantial (>30-fold) differences among these unstimulated groups, and were called present, were collected. This collection was hierarchically clustered according to relative expression level among the three cell types. Next, after exposure to *P. gingivalis* LPS, probesets showing a significant (based on one-way, six-level blocked analysis of variance; FDR, <10%), substantial (more than threefold) change in expression level in any one of the cell types were collected into a second group. Although the majority of these probesets showed a similar profile of behavior over all three cell types, increasing or more rarely, decreasing expression with *P. gingivalis* LPS exposure, some probesets showed changes to a greater or lesser degree in DCs or MAC than in MON. Probesets with differential expression (more than twofold) in monocytes and more than threefold more differential expression in the other cells, were separately identified. Conversely, probesets showing more than twofold differential in DCs or MAC, yet more than threefold greater differential expression in MON were also collected. These probesets distinguished the response profiles for each cell type.

Real-Time Polymerase Chain Reaction (PCR)

Total RNA (1 μg) from each sample was reverse-transcribed using an oligodeoxythymidylic acid primer (Invitrogen) and the resulting cDNA amplified by real-time PCR on an ABI Prism 7500 sequence detector (Applied Biosystems, Foster City, CA). Amplification was performed with TaqMan expression assays for HPRT (assay ID: Hs9999909_m1), β-actin (assay ID: Hs9999903_m1), tumor necrosis factor (TNF)-α (assay ID: Hs00174128_m1), CCL3 (assay ID: Hs00234142_m1), and SOCS4 (assay ID: Hs00377781_m1) from Applied Biosystems. HPRT and β-actin housekeeping genes were used for normalization controls. Amplification parameters and conditions were set by the manufacturer. Data were analyzed using the $2^{-\Delta\Delta CT}$ method[22] and results were reported as fold change.

Protein Expression Profiling

Eight independent experiments were performed with cells derived from eight healthy donors. Myeloid cell populations were cultured and stimulated as described above. After 24 hours with or without *P. gingivalis* LPS or *E. coli* LPS, the media were collected, spun at 12,000 $\times$ *g*, transferred to fresh tubes, and stored at -70°C. Supernatants were diluted fourfold in assay diluent (BioSource International, Camarillo, CA) before analysis using the Human Cytokine 25-Plex kit (Biosource, Invitrogen) and the Luminex 100 system (Luminex, Austin, TX). Data are expressed as mean $\pm$ SEM, compared using two-tailed Student's *t*-test for correlated samples (*http://faculty.vassar.edu/lowry/VassarStats.html*), and results considered significant at $P \leq 0.05$.

Results

Gene Expression in Inflamed Oral Mucosa and Infiltrating Myeloid Cells

In situ analysis of gingival tissues from patients with chronic periodontitis revealed the presence of large numbers of inflammatory cells compared with tissues from patients with noninflamed gingiva (Figure 1, A and B), and by preliminary microarray analysis, differential expression of multiple genes consistent with recruitment and activation of myeloid cell populations was evident (S. Nares, et al, manuscript in preparation). On further analysis of the inflamed tissues by immunohistochemistry, cells of myeloid lineage, including CD1a-positive dendritic cells (DCs) within the epithelium and within regions populated by inflammatory infiltrates (Figure 1C) and $CD68^+$ monocytes and macrophages (Figure 1, D and E) were seen. Monocytes were adherent to the endothelium (Figure 1D) and/or emigrated into the tissues (Figure 1E) within the inflammatory site. By comparison, healthy human gingival samples displayed minimal histological evidence of inflammatory infiltrates (Figure 1A), prompting our subsequent emphasis on the contribution of the myeloid cells to the inflammatory and tissue-destructive events characteristic of periodontal diseases.

Gene Expression in Myeloid Cell Populations

To elucidate the potential role of infiltrating myeloid cells in the defensive host response to periopathic bacteria

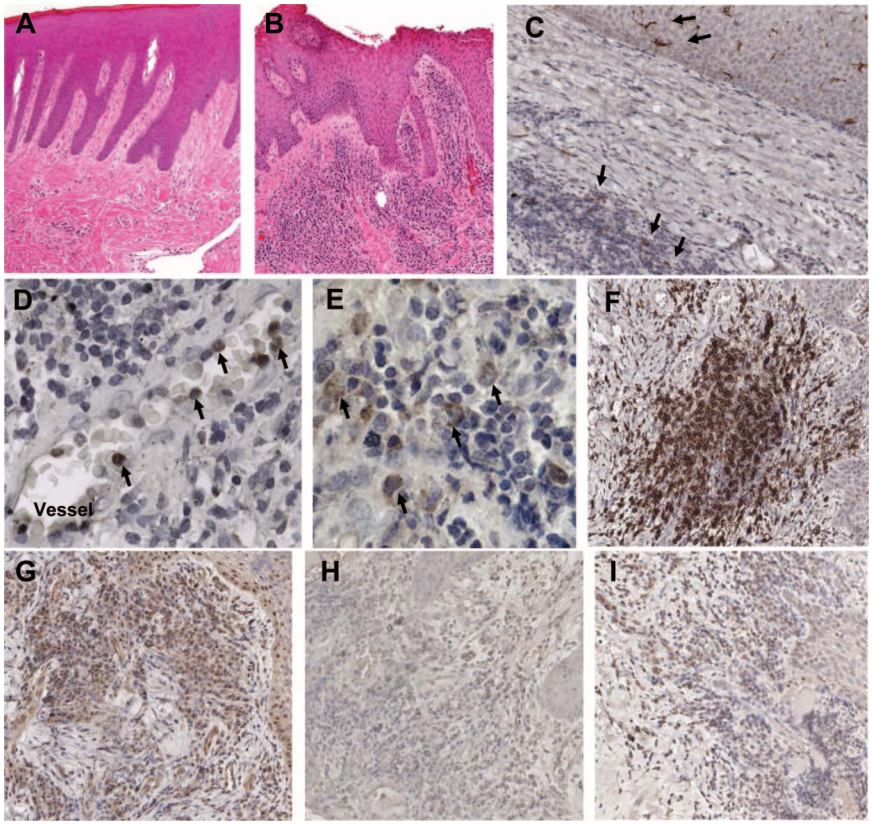

Figure 1. Histology and immunohistochemistry of gingival tissues. Biopsy specimens from healthy (**A**) and periodontally-involved (**B**) gingival tissues reveal dense inflammatory infiltrates in diseased specimens with minimal scattered accumulations of inflammatory cells in healthy samples (H&E). **C:** DCs in epithelium and in inflammatory infiltrates are stained with CD1a (**arrows**). **D** and **E:** Immunohistochemical staining of inflamed periodontal tissues with antibodies (CD68) that detect monocytes and macrophages (**arrows**). **F:** Staining of inflamed tissues with antibodies to T-cell-specific CD3. **G–I:** Staining of inflamed tissues adjacent to epithelium (right in each panel) with antibodies to IL-17 (**G**), IL-4 (**H**), and IFN-γ (**I**). Original magnifications: ×10 (**A**, **B**); ×20 (**C**, **G–I**); ×40 (**D**, **E**); ×20 (**F**).

and its evolution into a chronic destructive process, we compared the quantitative and qualitative responses of cells of myeloid lineage to *P. gingivalis*, considered a primary etiological agent in the initiation and persistence of periodontal disease. After isolation of human peripheral blood monocytes, the cells were used immediately as immature circulating monocyte populations or were differentiated into myeloid DCs and macrophages. We then compared the responses of the three maturationally distinct donor-matched populations to identical concentrations of TLR-activating *P. gingivalis* LPS, as a more uniform signal than the viable microbes. Demonstration of maturational status by flow cytometry revealed that phenotypically, monocytes were $CD14^+$, $HLA\text{-}DR^+$, $DC\text{-}SIGN^-$, $CD3^-$, and $CD19^-$, immature DCs were $CD1a^+$, $HLA\text{-}DR^+$, $DC\text{-}SIGN^+$, $CD86^+$, $CD14^{low}/^-$, $CD3^-$, $CD19^-$, whereas differentiated macrophages were $CD14^+$, $HLA\text{-}DR^+$, $CD68^+$, $CD3^-$, $CD19^-$, $DC\text{-}SIGN^-$ (Figure 2, A–C; CD14, CD68, and DC-SIGN shown).

After exposure to purified *P. gingivalis* LPS or an equivalent volume of PBS (control, unstimulated) for 1 hour, the early transcriptional responses of the three donor-matched cell populations were compared using oligonucleotide arrays. By activating the cells for only 1 hour, we focused on the primary TLR responses, minimizing downstream signaling effects and their potential differential consequences on gene transcription in each population. First, however, we noted a remarkable differential constitutive transcriptional profile among the three derivative populations, not attributable to donor variability. The unique subsets of genes detected in donor-matched unstimulated monocytes, DCs, and macrophages may distinguish the functional repertoire of these populations in host defense. To define these gene signatures more specifically, the most striking (30-fold or greater) differentially expressed probesets ($n = 184$) were hierarchically clustered (Figure 3, Table 1, and Supplemental Table S1 available at *http://ajp.amjpathol.org*). The cluster represented in red highlights those genes constitutively expressed at higher levels in the DCs [Figure 3, hierarchical and parallel plot cluster ($n = 3$ donors); Table 1, top 20 shown]. Notably, DCs independently expressed high levels of CD209 (DC-SIGN) and CD1, consistent with their phenotype, along with CD23A (IgE receptor), IL-17 receptor B, and histamine receptor (H1). Baseline enzymes, cathepsin C and arachidonate 15-lipoxygenase, also distinguished these cells from their myeloid counterparts. Constitutive expression of a myriad of CC chemokines (CCL8, CCL13, CCL14, CCL17, CCL18, CCL23, CCL24, CCL26) suggests that this population is at the ready to drive recruitment of innate effector cells on challenge with pathogens and antigens.

On the other hand, differentiated macrophages share a subset of baseline genes with DCs (Figure 3, orange cluster; and Supplemental Table S1 available at *http://ajp.amjpathol.org*): APOE, fibronectin, alpha2 macroglobulin, mannose receptor, succinate receptor, formyl peptide receptor-like 2/FPRL2, TREM 2, neuropilin, C1q, and CCL22), but uniquely (Figure 3, blue cluster; and Table 1) exhibit a repertoire of enzymes, including DNase, MMP7, MMP9, decysin, a disintegrin and metalloprotease (ADAM) family member linked to monocyte

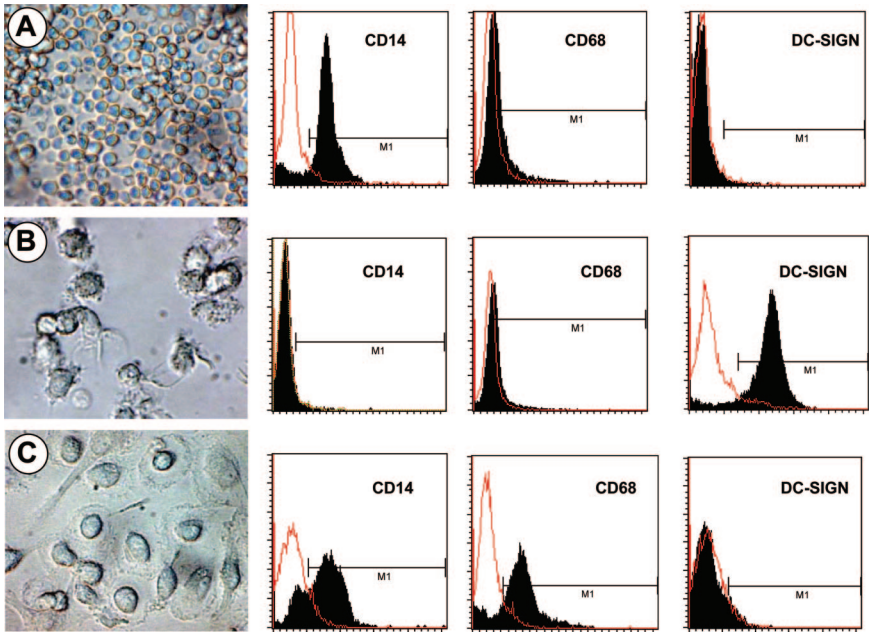

Figure 2. Phenotypic analysis of monocytes and differentiated DCs and macrophages. Freshly isolated peripheral blood monocytes (**A**), cultured and differentiated monocyte-derived DCs (**B**), and differentiated monocyte-derived macrophages (**C**) as shown in the **left** panels, were stained with the following fluorochrome-conjugated antibodies: CD14, CD68, and DC-SIGN and cellular phenotypes determined by flow cytometry analysis, **right** panels.

differentiation,[23] lipases, chitinases with innate anti-fungal activity toward chitin-containing fungi,[24] and ATPase (V0 subunit d2). Immature monocytes, freshly isolated from peripheral blood, and parental to DCs and macrophages, display their own profile of constitutively highly expressed genes (Figure 3, green cluster; and Table 1). Among the genes relatively overexpressed in immature monocytes were genes linked with innate immunity including several S100 calcium-binding proteins (S100A8, S100A9, S100A12), α1 defensin, Fcα receptor (CD89), selectin L, FPR1, and CXCR4. Other genes are distinctively enriched in resting immature monocytes, such as serpins, cyclin-dependent kinase inhibitor 1C (p57/Kip2), dual specificity phosphatase (DUSP)6, and properdin. Versican, a large chondroitin sulfate proteoglycan, associated with monocytes in wound healing, tissue remodeling, and myocardial infarcts[25] was more abundantly expressed in monocytes.

P. gingivalis-Inducible Gene and Protein Expression in Myeloid Cells

On exposure to *P. gingivalis* LPS, however, the three derivative donor-matched populations all responded with enhanced transcription of a plethora of pro-inflammatory genes, with 567 probesets showing demonstrable responses in at least one cell type (Supplemental Table S2 available at *http://ajp.amjpathol.org*). The use of donor-matched cells of different maturation levels ensured a less heterogeneous response to TLR signal-induced stimulation and in response to *P. gingivalis* LPS, a striking pattern of convergent gene expression of the three derivative myeloid populations suggested an early myeloid inflammatory transcriptional core response to *P. gingivalis* LPS. This response was clearly consistent with engagement of the TLR-NF-κB signaling network underlying expression of proinflammatory cytokines, anti-apoptotic factors, and antimicrobial molecules, crucial to an antimicrobial response of the host, and reflective of the ability of the purified preparation of *P. gingivalis* LPS that is enriched for two lipid A species (1435/1450) to activate TLR2, TLR2/1, and TLR4.[9]

Of the 567 *P. gingivalis* LPS-inducible probesets, modulation of molecules linked to intracellular signaling and regulation of NF-κB activity (NF-κBIA, NF-κBIZ, IRAK2, RIPK2, NF-κB2, EGR1, EGR3, DUSP1, DUSP2) (Supplemental Table S2 available at *http://ajp.amjpathol.org*) was consistent with TLR activation and induction of NF-κB-responsive genes, including induction of cytokine genes (IL-1α, IL-1β, IL-6, TNF-α, IFN-β1) and chemokines. Reflecting the up-regulation of TNF-α, TNF-α inducible genes/proteins were also rapidly up-regulated in cells exposed to *P. gingivalis* LPS (TNFAIP2, TNFAIP3, TNFAIP6, TNFAIP8). To corroborate the microarray expression profiling data, real-time PCR was performed for several representative genes found to be significantly influenced in response to *P. gingivalis* LPS, as shown for TNF-α (Figure 4A). Although the two assays are numerically not directly comparable, with RT-PCR being more sensitive, the directional magnitude of the increased expression levels was consistent. Moreover, when culture protein levels were monitored by multiplex bead assay, the striking increases in *P. gingivalis* LPS-induced TNF-α protein verified the gene induction, as well as the cell-specific responses of TNF-α expression (Figure 4B, log scale).

To demonstrate that intact *P. gingivalis* microorganisms, in addition to their cell wall-derived components, also influence these cells to generate the panoply of pathogenic inflammatory mediators, we exposed adherent monocyte-derived macrophages to viable bacteria. Addition of fluorescein isothiocyanate-tagged *P. gingivalis* to macrophage monolayers resulted in rapid binding to macrophage membranes (Figure 4C, inset), followed by

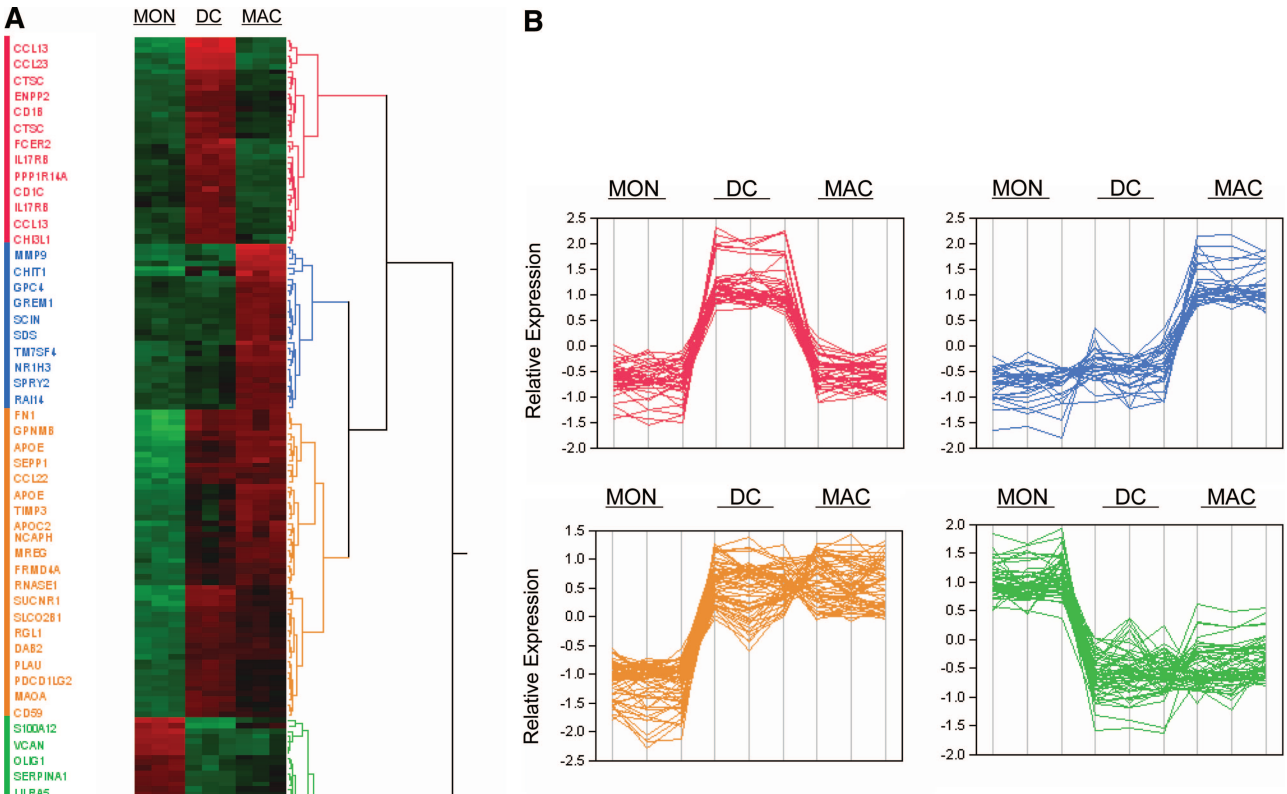

Figure 3. Hierarchical cluster of differentially expressed genes in three cell types. **A:** The microarray data were transformed and normalized using the S10 transform and probesets that are present (>50%) in one of the three groups and differ by greater than 30-fold among cell types were selected. Expression levels relative to mean for these 184 probesets were clustered according to Ward's method. Selected gene symbols are given (full list in Supplemental Table S1 available at *http://ajp.amjpathol.org*). **B:** Parallel line plots of relative expression of each probeset, in separate clusters reveal distinct patterns of expression in monocytes (MON), dendritic cells (DC), and macrophages (MAC).

internalization (Figure 4C). Furthermore, this encounter with intact bacteria resulted in induction of gene expression in the *P. gingivalis*-infected macrophages. By RT-PCR, TLR-dependent signaling resulted in transcriptional up-regulation of inflammatory cytokines, represented by TNF-α (Figure 4D), subsequent to NF-κB activation. Collectively, these data indicate that *P. gingivalis* and/or its endotoxin component can interact with myeloid cell populations to trigger transcription, production, and secretion of inflammatory mediators including chemokines, cytokines, and proteases, all of which may be instrumental in negotiating the path to chronic inflammation and tissue destruction characteristic of periodontal disease. Thus, this approach of exploring myeloid cell-induced gene and protein expression in response to *P. gingivalis* LPS *in vitro* provides reproducible data consistent with the presence and activation status of innate immune cells in inflamed gingiva in which *P. gingivalis* is considered a pivotal mediator of immunopathology.

Cell-Specific Responses to P. gingivalis LPS

In addition to the distinct differences in baseline/unstimulated transcriptional profiles between monocytes, DCs, and macrophages, and the core *P. gingivalis* LPS response across these populations, cell-specific gene regulation was also evident. The most dramatic of these are presented in Figure 5 in which the *P. gingivalis* LPS-induced gene profiles of DCs and macrophages are plotted against that of monocytes. In Figure 5A, MON *P. gingivalis* (Pg)LPS/C is plotted against DC PgLPS/C to differentiate probesets with more than threefold response in DCs (44 probesets, above green line) than MON, and probesets more than threefold in MON compared with DCs (eight probesets, below red line). A similar comparison between MON and MAC revealed 19 probesets greater in MAC than MON (Figure 5B, above blue line), whereas 16 probesets were higher in MON than MAC (below red line). Although the majority (60%) of the 567 differentially expressed probesets lie within the lines of identity (less than threefold difference between the two populations), those differentially expressed may be of consequence in the acute compared with chronic milieu of the inflamed gingiva. To further delineate shared and distinct maturation-dependent responses, a relative comparison across all three cell types for genes most differentially expressed at levels more than or equal to fivefold between at least two of the cell pop-

Table 1. Top 20 Constitutively Expressed Genes in Unstimulated Monocytes, Macrophages, and Dendritic Cells*

Monocytes	
S100 calcium-binding protein A8	*S100A8*
S100 calcium-binding protein A12	*S100A12*
Versican	*VCAN*
Defensin, α1	*DEFA1*
Ficolin (collagen/fibrinogen domain containing) 1	*FCN1*
Triggering receptor expressed on myeloid cells 1	*TREM1*
Hemoglobin, α2	*HBA2*
MRNA full-length insert cDNA clone EUROIMAGE 85905	
FBJ murine osteosarcoma viral oncogene homolog B	*FOSB*
Fc fragment of IgA, receptor for	*FCAR*
Hemoglobin, α1	*HBA1*
Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	*CDKN1C*
S100 calcium-binding protein A9	*S100A9*
Prokineticin 2	*PROK2*
Formyl peptide receptor 1	*FPR1*
Selectin L (lymphocyte adhesion molecule 1)	*SELL*
Myotubularin-related protein 11	*MTMR11*
Proplatelet basic protein (chemokine (C-X-C motif) ligand 7)	*PPBP*
Pre-B-cell colony-enhancing factor 1	*PBEF1*
Chromosome 19 open reading frame 59	*C19orf59*
Dendritic cells	
Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	*CCL18*
Chemokine (C-C motif) ligand 13	*CCL13*
Chemokine (C-C motif) ligand 26	*CCL26*
Chemokine (C-C motif) ligand 23	*CCL23*
Arachidonate 15-lipoxygenase	*ALOX15*
Chemokine (C-C motif) ligand 24	*CCL24*
Fc fragment of IgE, low affinity II, receptor for (CD23)	*FCER2*
CD209 molecule	*CD209*
Chemokine (C-C motif) ligand 17	*CCL17*
C-type lectin superfamily 4, member G	*CLEC4G*
Cathepsin C	*CTSC*
Interleukin 17 receptor B	*IL17RB*
CD1b molecule	*CD1B*
Forkhead box Q1	*FOXQ1*
Chemokine (C-C motif) ligand 14	*CCL14*
Receptor (G protein-coupled) activity modifying protein 1	*RAMP1*
Wingless-type MMTV integration site family, member 5B	*WNT5B*
Coagulation factor XIII, A1 polypeptide	*F13A1*
Chemokine (C-C motif) ligand 13	*CCL13*
Purinergic receptor P2Y, G-protein coupled, 14	*P2RY14*
Macrophages	
Chitinase 3-like 1 (cartilage glycoprotein-39)	*CHI3L1*
Matrix metallopeptidase 9 (gelatinase B, 92-kDa gelatinase, 92-kDa type IV collagenase)	*MMP9*
Matrix metallopeptidase 7 (matrilysin, uterine)	*MMP7*
Chitinase 1 (chitotriosidase)	*CHIT1*
Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	*SPP1*
ADAM-like, decysin 1	*ADAMDEC1*
Galanin prepropeptide	*GAL*
Deoxyribonuclease II β	*DNASE2B*
Cellular retinoic acid-binding protein 2	*CRABP2*
Transmembrane 7 superfamily member 4	*TM7SF4*
Similar to complement C3 precursor	*LOC653879*
Gremlin 1, cysteine knot superfamily, homolog (*Xenopus laevis*)	*GREM1*
Secretogranin V (7B2 protein)	*SCG5*
ATPase, H+ transporting, lysosomal 38-kDa, V0 subunit d2	*ATP6V0D2*
Glypican 4	*GPC4*
Scinderin	*SCIN*
Transcribed locus	
Collagen, type XXII, α1	*COL22A1*
Regulator of G-protein signaling 20	*RGS20*
Serine dehydratase	*SDS*

*Freshly isolated monocytes and differentiated donor-matched monocyte-derived macrophages and dendritic cells. Transcriptional profiles of unstimulated populations were determined by oligonucleotide arrays. Data represent the top 20 constitutively expressed genes in each myeloid population. Full expression patterns in the unstimulated cells are shown in Supplemental Table S1 available at *http://ajp.amjpathol.org*.

ulations after *P. gingivalis* LPS stimulation is summarized in Table 2.

DCs were the most transcriptionally responsive to *P. gingivalis* LPS with 434 probesets (Supplemental Table S2 available at *http://ajp.amjpathol.org*), whereas monocytes (270 probesets) and macrophages (224 probesets) responded with a somewhat reduced gene profile. Analysis of individual cellular responses revealed a number of uniquely

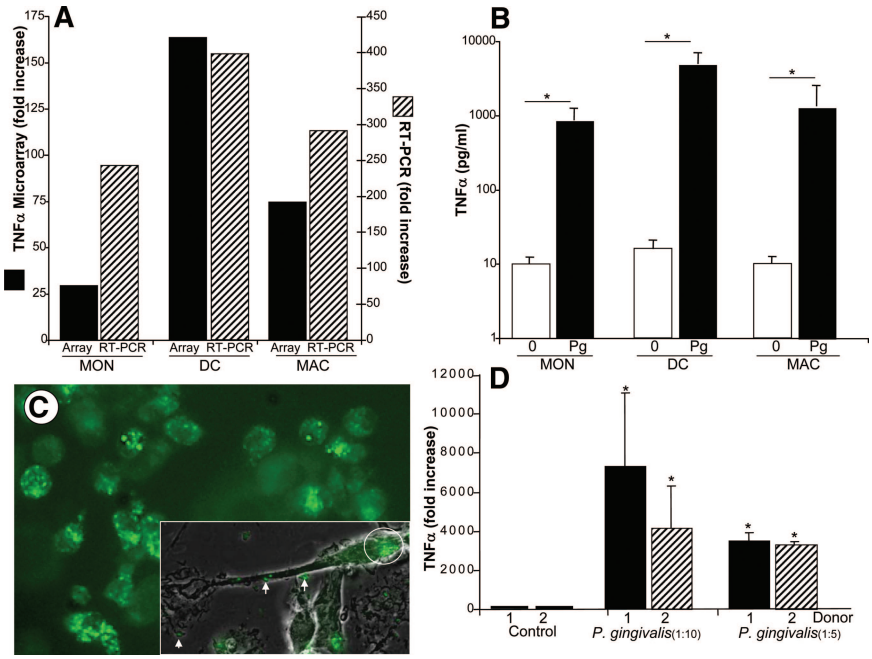

Figure 4. Corroboration of *P. gingivalis* LPS-induced TNF-α by microarray, RT-PCR, and protein. **A:** Populations of monocytes, DCs, and macrophages were exposed to *P. gingivalis* LPS (100 ng/ml) in parallel for 1 hour and the cells processed for microarray analysis and for real-time (RT)-PCR for TNF-α. Data represent fold increase based on *P. gingivalis* LPS/control (n = 3 donors). **B:** Cultures of monocytes, DCs, and macrophages were treated with PBS or *P. gingivalis* LPS for 24 hours and the supernatants collected and assayed for TNF-α by multiplex bead assay (mean ± SEM, n = 8 donors). $^*P < 0.05$. **C:** Intact *P. gingivalis* labeled with fluorescein isothiocyanate (100 μg/ml for 30 minutes) were added to macrophage monolayers and examined for bacterial binding at the membrane (**inset**, 5 minutes; **arrows** indicate membrane-associated, **circle** indicates internalized bacteria), and/or uptake by the macrophages (20 minutes). **D:** Macrophage cultures were exposed to live *P. gingivalis* at 10:1 or 5:1 for 1 hour and total RNA was extracted and processed for RT-PCR for TNF-α. Data represent fold increase for *P. gingivalis* LPS in two donors compared with control. $^*P < 0.05$. Original magnifications: ×63 (**C**, **inset**); ×32 (**C**).

expressed genes. After only 1 hour, 191 probesets were differentially expressed in DCs including REL and DUSP6, whereas 91 probesets such as IL-1RN and SOCS6 were distinct to monocytes. Interestingly, only 32 genes were found to be specific to *P. gingivalis* LPS-triggered macrophages. Ten transcripts were expressed both in monocytes and macrophages whereas 61 genes/ESTs were expressed in both monocytes and DCs. Macrophages and DCs expressed 74 transcripts exclusive of monocytes.

TLR2- and TLR4-Dependent Signaling

Whereas TLR2 signaling generates NF-κB-dependent activity, TLR4 engagement activates NF-κB and the interconnected transcription factor interferon regulatory factor (IRF)3 via TRAM-TRIF adaptors[26] with subsequent triggering of type I IFN. Because ligand interactions with TLR4, but not TLR2 trigger parallel, but separable pathways to influence both NF-κB and IFN-linked signal transduction, we examined the *P. gingivalis* LPS-induced transcriptional profile for potential TRIF adaptor-dependent gene induction, in addition to the evident NF-κB pathways. Some IFN-inducible genes were concurrently upregulated by *P. gingivalis* LPS, along with IFN-β1, which may contribute to an autocrine loop impacting on IFN-inducible genes as reported in other studies,[15] including guanylate binding protein 1, IFN-inducible (GBP1), GBP2, and IFN-induced protein with tetratricopeptide repeats 2 (IFIT2) (Supplemental Table S2 available at *http://ajp.amjpathol.org*). Moreover, these data suggest that TLR4 signaling pathways were engaged by *P. gingivalis* LPS. To directly compare a dominant TLR4-mediated response with the apparent TLR2/TLR4 signal induced by *P. gingivalis* LPS, we stimulated the three myeloid populations with *E. coli* LPS, a TLR4 agonist that engages both NF-κB and IRF3 pathways,[27] in parallel. The comparison of *E. coli* LPS (TLR4) and *P. gingivalis* LPS (TLR2/TLR4) revealed enhanced IRF3 activation and induction of IFN-β and related genes in those cells treated with *E. coli* LPS. In this regard, 12 of the top 20 significantly differentially expressed genes are linked to IFN pathways (Supplemental Table S3 available at *http://ajp.amjpathol.org*). Consequently, our data support a preferential, although not exclusive TLR2-NF-κB response for *P. gingivalis* LPS, along with the TLR4 signal.

In addition to transcription induction, the intensity and duration of TLR-induced responses are instrumental in the sequelae after TLR-mediated initiation of an inflammatory response meant to protect the host against pathogenic bacteria. In this regard, the transcriptional responses to equivalent amounts of *E. coli* LPS and *P. gingivalis* LPS vary considerably in intensity, with many of the *P. gingivalis*-induced genes (>90%) expressed at a reduced level compared with the parallel induction by *E. coli* LPS (pure TLR4 agonist) that has both MyD88-dependent and -independent effects on NF-κB and IRF3 activation and gene expression. This was also evident at the protein level as shown for representative pro-inflammatory chemokines and cytokines, CCL5/RANTES and IL-12 (Supplemental Figure S1 available at *http://ajp.amjpathol.org*).

P. gingivalis-Enhanced Expression of Chemokines

Taking a closer look at the *P. gingivalis*-inducible gene profile (Supplemental Table S2 available at *http://ajp.amjpathol.org*), the cell convergent response included the early induction of genes encoding chemokines (CCL3, CCL4, CCL5, CCL8/IL-8) at extremely high levels, particularly CCL20 (MAC:MON:DC, 528:266:231 fold increase). Rapidly enhanced expression of CXCL1, CXCL2, and CXCL3 (Gro α, β, γ), which engage CXCR2, is consistent

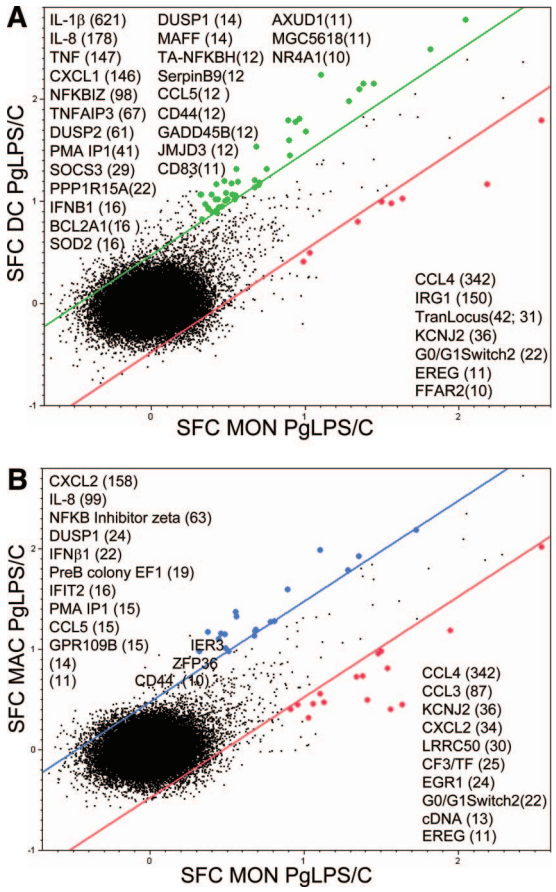

Figure 5. *P. gingivalis* induced gene expression in monocytes, DCs, and macrophages. **A:** The monocyte PgLPS/C response is plotted on the *x* axis and DC PgLPS/C on the *y* axis both in S10 (similar to log10) scale. The green line is threefold above the line of identity and the red line is threefold below. The green points (44 probesets) above the green line have more than twofold up-regulation in MON and more than threefold further up-regulation in DCs, whereas the red points (eight probesets) below the red line are more than twofold up-regulated in DCs and more than threefold further up-regulated in MON as shown in Supplemental Table S2 available at *http://ajp.amjpathol.org*. Only genes with >10-fold up-regulation above their respective unstimulated control population are listed in the figure, which does not include hypothetical genes, transcribed loci, and unidentified cDNA (see Table 2 for details). **B:** The monocyte PgLPS/C is plotted on the *x* axis and macrophage PgLPS/C on the *y* axis both in S10 scale. The blue line is threefold above the line of identity and the red is threefold below. The blue points (19 probesets) above the blue line have more than twofold up-regulation in MON and more than threefold further up-regulation in MAC whereas the red points (16 probesets) below the green line are more than twofold up-regulated in MAC and more than threefold further up-regulated in MON as shown in Supplemental Table S2 available at *http://ajp.amjpathol.org*. Only genes with >10-fold up-regulation above their respective unstimulated control population are listed in the figure, which does not include hypothetical genes, transcribed loci, and unidentified cDNA (see Supplemental Table S2 available at *http://ajp.amjpathol.org* for details).

with accumulation of myeloid cells. Although the magnitude of the response relative to individual chemokines varied between populations (Figure 5, Table 1, and Supplemental Table S2 available at *http://ajp.amjpathol.org*), the *P. gingivalis* induction of a chemokine transcriptional response was often >100-fold greater than the respective unstimulated control populations. This enhanced gene expression was reflected by secretion of multiple chemokines, as measured in the 24-hour supernatants of the *P. gingivalis* LPS-treated cultures (Figure 6, $n = 8$) (representative CCL and CXCL chemokines shown) with corresponding differences based on cellular differentiation. Chemokine receptor expression was not substantively altered in monocytes, DCs, or macrophages, with the exception of down-regulated CCR2 in monocytes, which may facilitate retention at the site of inflammation. Moreover, augmented expression of genes that enhance adhesion and migration (CD44, CD54/ICAM1) was seen in the stimulated cells. Enhanced tissue factor (coagulation factor III) (MON:DC:MAC, 25:54:3) may be geared toward promoting a local coagulation cascade to limit microbial dissemination.

Cytokines and Co-Stimulatory Molecules Associated with Innate/Adaptive Immunity

Cytokines with pro-inflammatory activities and linked to engaging adaptive immunity and driving Th1 and Th17 lineage commitment, including IL-1, IL-6, IL-12, and IL-23 (Figure 5; Figure 7, A–D; and Supplemental Table S2 available at *http://ajp.amjpathol.org*) increased in all three myeloid populations, albeit at quantitatively varying levels. Whereas *P. gingivalis* LPS up-regulated expression of the shared IL-12 and IL-23 p40 in all three populations, the IL-23-specific p19 was typically increased to a greater extent than IL-12p35 (Figure 7, C and D). In addition to elevated levels of myeloid IL-1, IL-23 and IL-6, COX2 (MON:DC:MAC, 24:65:53) has also been linked to Th17 lineage commitment.[28] Cytokine transcription was linked to the secretion of these inflammatory mediators as determined in 24-hour supernatants from myeloid cells derived from eight healthy donors tested in a multiplex bead assay. Reflecting the differential responsiveness at the transcriptome level, protein levels for the evaluated cytokines varied among these myeloid populations, as shown for IL-lβ, IL-6, and also TNF-α (Figure 3 and Figure 7, A and B). However, in keeping with the requirement for posttranscriptional inflammasome-mediated processing of the IL-lβ precursor protein,[29] macrophages and DCs secreted little active IL-lβ protein (Figure 7A), despite $\geq$100-fold increases in gene expression (Figure 7A and Supplemental Table S2 available at *http://ajp.amjpathol.org*). Thymic stromal lymphopoietin (TLSP), selectively induced more than fourfold in macrophages in response to *P. gingivalis* LPS, is considered a factor indirectly involved in Th2 cell generation[30] and only monocytes up-regulated their expression of IL-18 (more than threefold), a Th1-biasing cytokine. In addition to the dominant pro-inflammatory cytokines, which suggest a bias toward Th17 lineage polarization, several co-stimulatory molecules were also up-regulated in the derivative myeloid cell populations, including CD80, CD83, CD44, CD69, CD58/LFA-3. The innate cell response to *P. gingivalis* provides instructions for localization and lymphocyte effector cell type differentiation at the site of infection. Functional links between innate and adaptive responses to infection are forged through TLR, but interestingly, no increased transcription of TLR (1 hour) or significant change in cell surface expression levels of TLR2 or TLR4 (24 hours) were observed after challenge with *P. gingivalis* LPS (data not shown).

Table 2. Relative Differences in *P. gingivalis*-Induced Gene Expression in Myeloid Cell Populations

Gene title	Gene symbol	Myeloid population		
		MON	DC	MAC
Potassium inwardly-rectifying channel, subfamily J, member 2	*KCNJ2*	13.7*	3.7	1.0
Immunoresponsive 1 homolog (mouse)	*IRG1*	9.9	1.0	5.0
Interleukin 1 receptor antagonist	*IL1RN*	9.6	1.6	1.0
Coagulation factor III (thromboplastin, tissue factor)	*F3*	7.9	17.0	1.0
Free fatty acid receptor 2	*FFAR2*	7.3	2.0	1.0
Gap junction protein, β2, 26 kDa	*GJB2*	6.4	4.9	1.0
Chemokine (C-C motif) ligand 3	*CCL3*	5.6	2.7	1.0
Chemokine (C-C motif) ligand 4	*CCL4*	5.3	1.0	1.7
Chemokine (C-X-C motif) ligand 2	*CXCL2*	5.2	2.1	1.0
Epiregulin	*EREG*	4.9	1.5	1.0
Interleukin 8	*IL8*	1.0	14.3	7.9
Jumonji domain containing 3, histone lysine demethylase	*JMJD3*	1.0	11.3	2.4
Dual specificity phosphatase 2	*DUSP2*	1.4	10.4	1.0
Arrestin domain containing 3	*ARRDC3*	1.0	10.1	3.2
BCL2-related protein A1	*BCL2A1*	1.8	9.8	1.0
Inhibin, βA	*INHBA*	1.0	9.6	1.2
Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, ζ	*NFKBIZ*	1.0	8.4	5.3
v-fos FBJ murine osteosarcoma viral oncogene homolog	*FOS*	1.5	8.1	1.0
v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	*MAFF*	2.4	7.5	1.0
Phorbol-12-myristate-13-acetate-induced protein 1	*PMAIP1*	1.0	7.5	3.3
Tumor necrosis factor, α-induced protein 3	*TNFAIP3*	1.0	7.4	2.0
Superoxide dismutase 2, mitochondrial	*SOD2*	1.0	7.0	2.3
Growth arrest and DNA damage-inducible, β	*GADD45B*	1.0	7.0	4.2
Immediate early response 2	*IER2*	1.0	7.0	1.9
Interferon regulatory factor 1	*IRF1*	1.0	6.9	3.0
Tumor necrosis factor (ligand) superfamily, member 9	*TNFSF9*	1.0	6.8	4.0
RasGEF domain family, member 1B	*RASGEF1B*	1.0	6.4	2.1
Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, α)	*CXCL1*	1.0	6.2	2.7
Dual specificity phosphatase 1	*DUSP1*	1.0	6.0	6.9
CD44 molecule (Indian blood group)	*CD44*	1.0	5.9	4.8
Interleukin 1, β	*IL1B*	1.0	5.8	1.1
Regulator of G-protein signaling 1	*RGS1*	1.0	5.6	1.7
DNA damage-inducible transcript 4	*DDIT4*	3.7	5.6	1.0
T-cell activation NFKB-like protein	*TA-NFKBH*	1.5	5.5	1.0
Activating transcription factor 3	*ATF3*	1.3	5.5	1.0
Tumor necrosis factor (TNF superfamily, member 2)	*TNF*	1.0	5.5	2.5
Interleukin 7 receptor	*IL7R*	1.0	5.3	3.2
Mitogen-activated protein kinase kinase 3	*MAP2K3*	1.0	5.3	1.2
Chemokine (C-C motif) ligand 8	*CCL8*	1.0	5.2	1.9
Rap guanine nucleotide exchange factor (GEF) 2	*RAPGEF2*	1.0	5.0	2.9
Pre-B-cell colony-enhancing factor 1	*PBEF1*	1.0	3.7	7.1
G protein-coupled receptor 109B	*GPR109B*	1.0	1.8	6.6
Interferon, β1, fibroblast	*IFNB1*	1.0	4.5	6.1
Chemokine (C-C motif) ligand 5	*CCL5*	1.0	3.3	5.2
B-cell CLL/lymphoma 11A (zinc finger protein)	*BCL11A*	1.0	4.7	5.0
Interferon-induced protein with tetratricopeptide repeats 2	*IFIT2*	1.4	1.0	4.7

*Relative changes in gene expression. The cell population expressing the lowest *P. gingivalis* LPS-induced fold change was set at one 1, and the values for the other two myeloid cell populations represent their increase in gene expression relative to the population with the lowest response to *P. gingivalis* LPS. Only genes with more than or equal to fivefold differential compared with the least responsive cells are shown. Hypothetical proteins, cDNA, transcribed loci, cDNA clones, and replicate probsets are excluded from the list.

Detection of Th Lineages in Inflamed Periodontal Tissues

Based on our *in vitro* data incriminating myeloid cells and their products in periodontal pathogenesis, we further validated our findings by assessing whether these *P. gingivalis*-induced genes were reflected by potential functional consequences in the inflamed tissues. The abundance of myeloid cell chemokines (CCL20) and enhanced cytokines implicated in Th17 recruitment and polarization (IL-1, IL-6, IL-23), were consistent with their involvement in combating bacterial pathogens[31] and reflected evidence for IL-17-secreting cells (Figure 1G) within the T cell ($CD3^+$) compartment of the inflammatory lesions (Figure 1F). IFN^+ (Th1) cells were identified (Figure 1I), consistent with antigen-presenting cell IL-12 gene and protein expression (Figure 7C and Supplemental Figure S1 available at *http://ajp.amjpathol.org*), with lesser evidence for Th2 ($IL\text{-}4^+$) (Figure 1H) cell accumulation.

Mediators of Tissue Destruction and Bone Resorption

Multiple factors known to impact on osteoclast formation and function were identified in response to *P. gingivalis* LPS, implicating innate immune cells from the very onset of the response in the subsequent alterations in bone

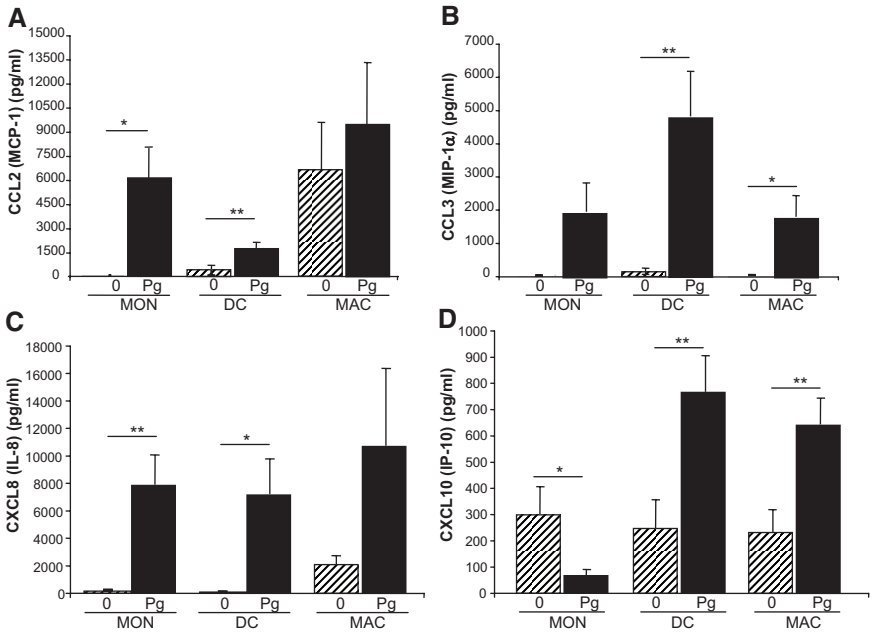

Figure 6. Chemokine protein response after exposure to *P. gingivalis* LPS. **A–C:** Monocytes (MON), macrophages (MAC), and dendritic cells (DC) were incubated with *P. gingivalis* LPS for 24 hours and supernatant levels of indicated chemokines assayed by multiplex bead assay, demonstrating significantly elevated levels of CCL2 (**A**), CCL3 (**B**), CXCL8 (**C**), and CXCL10 (**D**) as part of the proteomic response to *P. gingivalis* LPS, which varies quantitatively among the three derivative populations ($n = 8$ donors, two-tailed Student's *t*-test for correlated samples). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

metabolism. However, with differentiation and independently of *P. gingivalis* LPS, monocyte-derived macrophages and DCs acquired elevated expression of MMP, TREM2, CD44, purinergic receptor P2X (P2RX7), and ATPase (ARP6V0D2) a component of the V-type H^+ ATP6i proton pump complex that secretes H^+ from osteoclasts[32] (Figure 3 and Supplemental Table S1 available at *http://ajp.amjpathol.org*), consistent with their role as precursors to osteoclastogenesis in the appropriate microenvironment. On *P. gingivalis* activation, myeloid cells augment their repertoire of inducers/activators (TNF-α, IL-1β, IL-6, COX2) at the gene and protein level (Figures 4 and 7), which not only activate existent osteoclasts, but have the potential to drive macrophages and DCs into the multinucleated bone resorbing osteoclast phenotype, likely supported by IL-17 and T-cell receptor activator of NF-κB ligand (RANKL) engagement of osteoclast RANK.[33] Extracellular matrix-degrading proteases (MMPs) are increased without an apparent corresponding increase in TIMP, an imbalance supporting exacerbation of the pathogenesis of periodontitis. *P. gingivalis* LPS also induced the expression of proteases

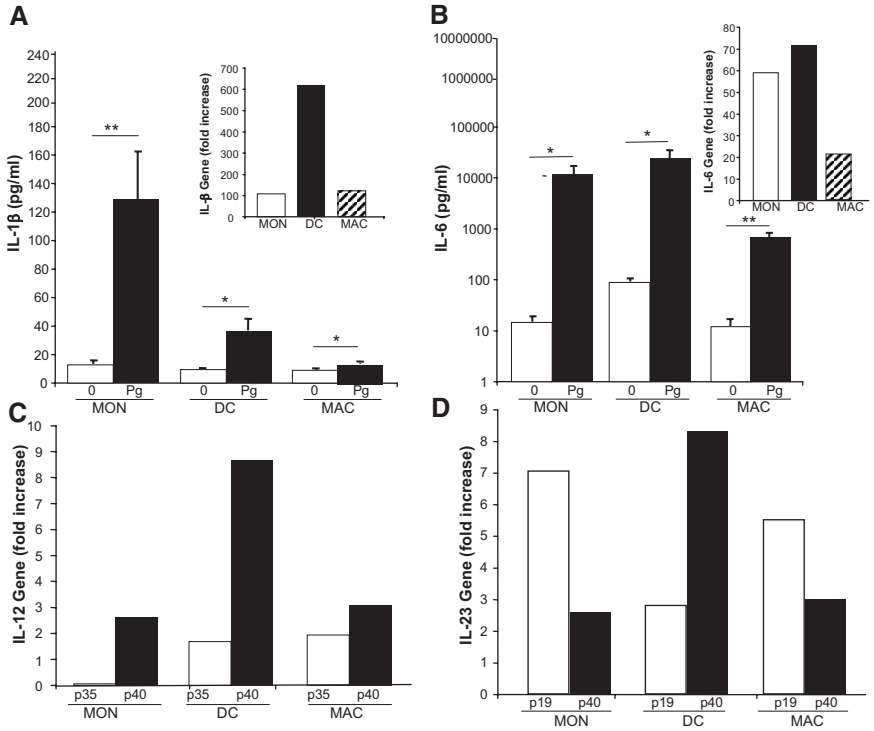

Figure 7. *P. gingivalis* LPS-induced inflammatory cytokines linked to T-lineage commitment. **A** and **B:** Monocytes (MON), macrophages (MAC), and dendritic cells (DC) were incubated with *P. gingivalis* LPS for 24 hours and supernatant levels of IL-lβ (**A**) and IL-6 (**B**) were determined by multiplex bead assay ($n = 8$ donors, two-tailed Student's *t*-test for correlated samples). **Insets:** Populations of monocytes, DCs, and macrophages were exposed to *P. gingivalis* LPS (100 ng/ml) in parallel for 1 hour and the cells processed for microarray analysis. Data represent fold increase based on *P. gingivalis* LPS/control ($n = 3$ donors) for IL-1β (**A**, **inset**) and IL-6 (**B**, **inset**). **C:** Populations of monocytes, DCs, and macrophages were exposed to *P. gingivalis* LPS (100 ng/ml) in parallel for 1 hour and the cells processed for microarray analysis. Data represent fold increase based on *P. gingivalis* LPS/control ($n = 3$ donors) for IL-12. Based on gene expression levels, DCs and macrophages expressed higher levels of IL-12A (p35) than monocytes, and DCs exhibited highest levels of IL-12B (p40) induced by *P. gingivalis* LPS within 1 hour. **D:** Comparison of IL-23 p19 and p40 gene expression in the three myeloid cells populations revealed that whereas DCs expressed higher levels of p40, monocytes and macrophages expressed more p19. $^{*}P < 0.05$; $^{**}P < 0.01$.

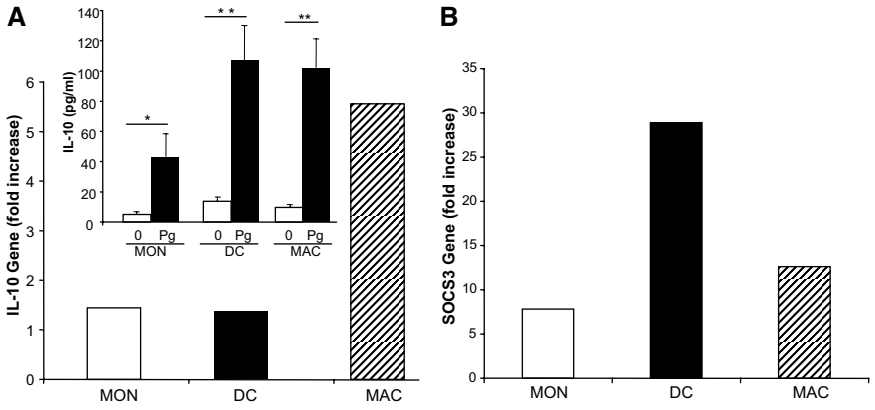

Figure 8. Induction of immunosuppressive molecules. **A** and **B:** Monocytes, DCs, and macrophages were exposed to *P. gingivalis* LPS (100 ng/ml) in parallel for 1 hour and the cells processed for microarray analysis. Data represent fold increase based on *P. gingivalis* LPS/control ($n = 3$ donors) for IL-10 (**A**) and SOCS3 (**B**). **A**, **inset**: Monocytes (MON), macrophages (MAC), and dendritic cells (DC) were incubated with *P. gingivalis* LPS for 24 hours and supernatant levels of IL-l0 were determined by multiplex bead assay ($n = 8$ donors, two-tailed Student's *t*-test for correlated samples). $^{*}P < 0.05$, $^{**}P < 0.005$.

(PLAU) and limited enhancement (two- to fourfold increase), depending on cell type, of serine protease inhibitors (SERPINB 1, 2, 8, 9) (Supplemental Table S2 available at *http://ajp.amjpathol.org*). Osteoclasts themselves are TLR^{+} and *P. gingivalis* may affect bone loss by directly engaging their NF-κB-dependent activation pathways in conjunction with complex cytokine regulatory effects related to their stage of differentiation.[34]

P. gingivalis Regulation of Immunosuppressive Molecules

Of interest is the limited evidence of an emergent suppressive response, with modest increases in IL-10 (fivefold) gene expression in the more differentiated macrophages, and modest protein secretion in all three populations (Figure 8A). Expression of suppressor of cytokine signaling (SOCS)3, which depending on probeset, was elevated in all three populations from 8- to 28-fold (Figure 8B) may dampen cytokine responsiveness. IL-1R antagonist (IL-1RN) was independently up-regulated 10-fold in stimulated monocytes (Table 2). A loss of IFN-γR1 expression was evident in all three populations subsequent to exposure to *P. gingivalis* LPS (three- to fivefold decrease) (Supplemental Table S2 available at *http://ajp.amjpathol.org*), likely minimizing IFN-γ-mediated responsiveness, and notable was the lack of up-regulation of TLR expression. Inhibitors of NF-κB, IκBα, IκBε, and IκBζ increased to varying degrees. Nonetheless, these several potential avenues for inhibition would appear to have limited impact in counteracting the pro-inflammatory profile and/or initiating resolution. Although transforming growth factor (TGF)-β was not transcriptionally elevated by *P. gingivalis* LPS, DCs constitutively express higher levels of TGF-β than monocytes or macrophages (data not shown), whereas the TGF-β family member, inhibin βA, was increased >17-fold in DCs, 2-fold in macrophages, but not increased in monocytes (Supplemental Table S2 available at *http://ajp.amjpathol.org*) in response to challenge with *P. gingivalis* LPS.

Another potential regulatory pathway may involve microRNA induction. The BIC transcript, a noncoding gene containing microRNA-155(miR-155) was uniquely induced by *P. gingivalis* LPS in all populations (MON:DC:MAC, 23:70:13-fold) (Supplemental Table S2 available at *http://ajp.amjpathol.org*). MiR-155 reportedly targets transcripts coding for several proteins involved in LPS signaling, Fas-associated death domain protein (FADD), IκB kinase ε, and TNFR superfamily-interacting serine-threonine kinase 1(Ripk1), while concomitantly enhancing TNF-α translation.[35] Through such mechanisms, BIC could play a pivotal role in posttranscriptional modulation of gene regulation by binding to complementary sequences within the 3′ untranslated region of target genes, evoking degradation or translational inhibition[36] (L. Strat and S. Nares, manuscript in preparation).

A significant number of poorly characterized genes and ESTs were also induced by *P. gingivalis* LPS (168 of 567, 29%). Among the top 50 most differentially expressed genes in monocytes, 12 (24%) are poorly characterized, whereas in DCs 5 (10%) and in macrophages 7 (14%) have yet to be characterized. Determining the potential role of these genes in the host response will likely contribute to the understanding of periodontal pathogenesis and other chronic inflammatory diseases and reveal unexplored therapeutic targets.

Discussion

Long-lived myeloid inflammatory cells such as macrophages and DCs, strategically poised along portals of entry, sample the local microenvironment in search of PAMPs.[20] On PAMP recognition, initiation of innate responses through generation of an arsenal of genes that encode effector proteins ensues to contain and/or clear the inciting agent, with a striking reprogramming of the complex molecular and cellular networks within and between immune cells. As coordinators, these cells orchestrate the transition to and the propagation of the adaptive arm of the immune response. Aberrancies in one or more of these processes may lead to a chronic state of inflammation or infection. During gingival irritation, ulceration of the epithelial barrier can provide a portal of entry for pathogenic bacteria and/or their products such as those derived from the periodontopathogen, *P. gingivalis* providing access to TLR^{+} immune cells.

Based on the early transcriptional profile in TLR^{+} myeloid cells, *P. gingivalis* LPS delivers a potent pro-inflammatory signal without apparent corresponding down-reg-

ulatory or anti-inflammatory gene expression. Despite similarities in the transcriptional and proteomic profiles of monocytes, DCs, and macrophages, we observed clear differences in the extent and magnitude of their responses, with DCs not only displaying embellished transcriptional activity, but typically secreting higher levels of cytokines and chemokines compared with their donor-matched monocytes and macrophages. After *P. gingivalis*-TLR initiation of transcription, processing of transcripts, their transport to cytoplasm, and translation of mRNA with protein synthesis and secretion, all contribute to the cellular responses to this pathogen. In this context, the transcription factor NF-κB plays a pivotal role in the innate TLR2/TLR4 response to *P. gingivalis*. In addition to NF-κB, the IRF3 pathway is engaged by *P. gingivalis* LPS, consistent with a TLR4 response,[27] albeit to a lesser degree than an exclusive TLR4 signal. This parallel pathway, by coordinately regulating IFN and IFN-related genes in the *P. gingivalis* LPS-stimulated myeloid populations, may in turn, regulate functions of innate cells including CTL and NK cells, as well as the transition into an adaptive response.

After TLR activation, IκB phosphorylation by IκB kinase (IKK) and its ubiquitin-dependent degradation liberate NF-κB for nuclear translocation and regulation of target gene transcription. Thus, a key event connecting TLR engagement to NF-κB activation is regulation of IKK activity, controlled by opposing actions of kinases and phosphatases[37] and *P. gingivalis* LPS-induced protein phosphatase regulatory inhibitor subunit 15A(PP1R15A) may temper the ability of the cells to control this pathway by disabling phosphatase inactivation of IKK.[38] Moreover, PPP1R15A was recently linked to responsiveness to TNF inhibitor therapy in rheumatoid arthritis.[39] Among the genes induced by *P. gingivalis* LPS is IκBα, which can re-exert control over NF-κB, along with IκB ϵ and particularly, IκB ζ, which binds to NF-κB p50 in the nucleus and has both negative (IL-8, TNF-α) and positive (IL-6, IL-12p40, GM-CSF, G-CSF) regulatory actions.[40,41] Although rapid NF-κB activation is necessary for host defense against microbial invasion, failure to re-exert control with persistent NF-κB activation may result in tissue injury, and in the extreme, to organ failure, systemic disease, and death.[42,43]

NF-κB-responsive genes, including abundant chemokines, were up-regulated both transcriptionally and at the protein level, consistent with rapid mobilization and accumulation of innate and/or adaptive immune cells at the site of *P. gingivalis* infection and release of cell wall components. Most dramatic chemokine expression was a feature of DCs, likely the first line of defense in the oral epithelium and arbiters of mucosal immunity and tolerance.[44] Oral lymphoid-like follicles containing $CD4^+$, $CD45RA^+$, and $CD45RO^+$ T cells in close proximity to immobilized mature $CD83^+$ DCs and B-cells situated within the gingival lamina[10] may represent an overzealous DC-dependent T-cell response. In particular, elevated levels of CCL20, observed in *P. gingivalis* LPS-stimulated myeloid cells and in inflamed periodontal tissues,[45] through its ability to attract $CCR6^+$ memory T cells, especially Th17 populations[46] and immature DCs, may continue to refuel the inflammatory response. In a murine model of arthritis, a significant correlation existed between IL-17 and CCL20 in diseased joints.[47]

Polarized with the assistance of APC, effector T cells were previously considered to differentiate into Th1 or Th2 phenotypes,[48] but with the recent recognition of the Th17 lineage, instrumental in antimicrobial immunity and in inflammatory and autoimmune pathologies,[49,50] there has been renewed interest in these cells in chronic mucosal lesions. *P. gingivalis*-activated cells not only generated IL-12, a facilitator of Th1 cells, but also IL-23, IL-1, and IL-6, all of which support Th17 polarization, either independently, or more likely in conjunction with TGF-β in humans,[51–53] as shown in rodents.[54] Delineating a role for Th1/Th2/Th17 lineages in periodontitis is ongoing,[55] but clearly the relevant instruction molecules are abundant to coordinate Th17, as well as Th1 lineage commitment. Whereas some reports indicated a paucity of Th1-inducing IL-12,[56] we noted a significant increase in IL-12p35 and p40 expression and in secreted IL-12p40/p70 protein in response to *P. gingivalis* LPS. Although we detected *P. gingivalis*-induced IL-10, as reported,[57] levels of IL-12 were consistently higher. Importantly, increased *P. gingivalis*-induced expression of heterodimeric IL-23, composed of p40 and p19, and connected to Th17 survival, were consistent with pro-inflammatory $IL\text{-}17^+$ cells within the lesions and inflamed gingiva.[58] IL-17 in turn accelerates innate cell recruitment and activation through IL-8, CXCL1, TNF, GM-CSF, and facilitates osteoclastogenesis.[59]

One of the hallmarks and most devastating aspects of periodontal disease is the destruction to the supporting structures of the tooth, involving osteoclastogenesis and bone resorption. Osteoclasts are formed by fusion of precursor cells of the monocyte/macrophage lineage,[60] and $CD11c^+$ DCs contribute to the pool of osteoclasts.[44,61] Beyond the direct effects of myeloid cell products on osteoprogenitors, Th17 cells have also been incriminated in the dynamics of bone resorption.[33,59] Potentially relevant is the up-regulation of IL-7R expression in macrophages and DCs by *P. gingivalis* in light of recent evidence that IL-7 enhances osteoclastogenesis and directly sensitizes T cells to release pro-osteoclastogenic factors including TNF-α and RANKL.[62] Evidence also supports a direct TLR signaling pathway on TLR^+ osteoclasts, promoting differentiation and survival, as well as signaling of TLR^+ osteoblasts to generate RANKL and TNF-α to further drive osteoclastogenesis.[34]

Our findings reveal the existence of an early aggregate response, which may reflect a convergence profile within an inflammatory site where all three lineage-specific populations accumulate, co-exist, and respond to pathogenic triggers. Their shared, unique and/or complementary transcriptional and proteomic profiles may frame the context of the host response to *P. gingivalis*, contributing to the destructive nature of periodontal inflammation. Moreover, the microbial flora of the oral cavity provides a source of multiple PAMPs with the potential to activate many, if not all, TLRs, along with host-derived molecules released by damaged cells in the chronic lesion that also activate TLRs. The repeated pathogen-induced onslaughts of acute inflammatory mediators in the context of

a chronic nonresolving lesion coalesce into a pro-inflammatory protease-laden destructive milieu. Beyond local responses to oral infection with *P. gingivalis*, emerging evidence supports systemic links to atherosclerosis, diabetes, and obesity,[7,63] along with evidence that infection-mediated increases in mucosal permeability[64] can eventuate in endotoxemia-induced inflammation distally. Continued dissection of the imbalance between inflammatory pathways and immunosuppressive elements may reveal strategies to blunt chronicity and destructiveness of pathogen-induced inflammation within the oral cavity and also systemically, being that elimination of the inciting agent(s) represents a formidable task.

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